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Cardiac Fibroblasts Produce Leukemia Inhibitory Factor and Endothelin, Which Combine to Induce Cardiac Myocyte Hypertrophy In Vitro

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Cardiac fibroblasts in culture produce factor(s) that induce hypertrophy of neonatal rat ventricular myocytes in vitro. As in vivo, the myocyte hypertrophy response in culture is characterized by an increase in cell size and contractile protein content, and by the activation of embryonic genes, including the gene for atrial natriuretic peptide. The purpose of this study was to identify the factor(s) produced by fibroblasts that induce myocyte hypertrophy. The fibroblast hypertrophy activity was inhibited using a combination of the endothelin A receptor blocker BQ-123 and an antibody to leukemia inhibitory factor. The individual antagonists each caused a partial inhibition. The mRNAs for both leukemia inhibitory factor and endothelin were detected by RT-PCR analysis and the concentration of both proteins was determined to be approximately 200 pmol/L in the conditioned medium using immunoassays. Purified leukemia inhibitory factor and endothelin each induced distinctive morphological changes in the myocytes. Their combination generated a different morphology similar to that induced by fibroblast conditioned medium. Each factor also induced atrial natriuretic peptide production, but both were required for the myocytes to produce the levels measured after exposure to fibroblast conditioned medium. These results show that hypertrophy activity produced by cardiac fibroblasts in culture is a result of leukemia inhibitory factor and endothelin.

Key Words: Cardiac fibroblasts; hypertrophy; leukemia inhibitory factor; endothelin.

Introduction

Hypertrophy of adult cardiac ventricular myocytes in vivo is a response to a variety of conditions that lead to chronic overload. Adult myocyte hypertrophy is initially beneficial as a short-term response to impaired cardiac function by permitting a decrease in the load on individual muscle fibers. With severe, long-standing overload, however, the hypertrophied cells begin to deteriorate and die (Katz, 1989). The signals that initiate hypertrophy in vivo are poorly understood. Possible mediators include stretch-activated ion channels, fibroblast growth factor, transforming growth factor β, α and β adrenergic agonists, angiotensin II, thryoxine, insulin, growth hormone, and glucocorticoids (Morris, 1990; Schnieder et al., 1990). These factors may be acting via autocrine, paracrine or endocrine routes.

The nonmyocyte component of the myocardium represents about two thirds of the cells in the heart and consists primarily of endothelial cells, smooth muscle cells, macrophages and fibroblast/mesenchymal cells (Nag, 1980). The contribution of paracrine factors produced by these cells to the development of hypertrophy of the cardiac muscle cells is difficult to determine in vivo because of the heterogeneity of the cell types in the heart and the complexity of extracellular signals. Several years ago a neonatal rat ventricular myocyte culture system was developed as an in vitro model for cardiac hypertrophy (Simpson et al., 1982). The myocyte hypertrophy response in culture shares several characteristic features of the in vivo response: increase in cell surface area/volume, increase in protein content/cell, accumulation and assembly of individual contractile proteins into organized sarcomeric units, activation of a distinct early gene program, transcriptional activation of constitutively expressed contractile protein genes and embryonic genes (most consistently the gene for atrial natriuretic peptide [ANP]), and lack of inducibility of a subset of muscle-specific genes (Chien et al., 1991). Endothelin-1, alpha-adrenergic agonists such as phenylephrine, serum, leukemia inhibitory factor (LIF) and the recently cloned cytokine cardiotrophin 1 induce hypertrophy in this system (Shubieta et al., 1990; Ito et al., 1991; Suzuki et al., 1991; Pennica et al., 1995).

Conditioned medium from cultured neonatal rat cardiac nonmyocytes (primarily fibroblast-like cells) stimulates the

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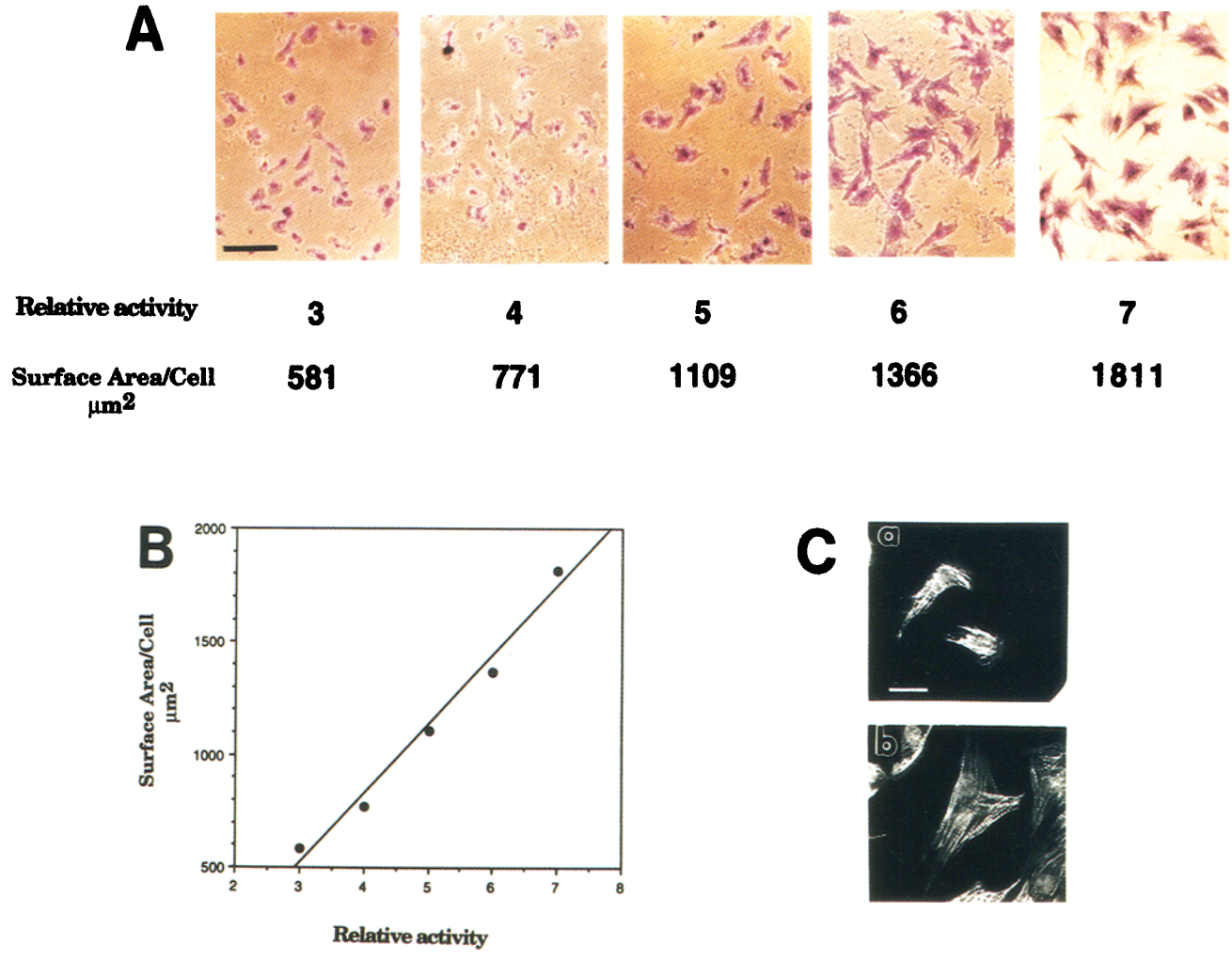

Fig. 1. Relative activity rating system and its correlation with myocyte surface area. Rat neonatal cardiac myocytes were treated with increasing concentrations of phenylephrine for 48 h and stained with crystal violet. **(A)** Stained myocytes wtih corresponding relative activity rating and mean surface area/cell. **(B)** Correlation of relative activity rating with surface area/cell. Correlation coefficient = 0.99. Areas of 50 myocytes/rating were measured. **(C)** Myocytes not treated (a) or treated (b) with 100 μmol/L phenylephrine for 48 h were stained with fluorescent phallicidin to show the f-actin in the contractile fibrils. (Panel A bar: 100 μm). (Panel C bar: 20 μm).

neonatal rat myocytes in culture to hypertrophy (Long et al., 1991). Partially purified activity had the characteristics of a protein and bound to heparin-sepharose. Experiments with neutralizing antisera to platelet-derived growth factor, tumor necrosis factor alpha, acidic and basic fibroblast growth factors, and transforming growth factor beta 1, eliminated these growth factors as possible candidates (Long et al., 1991).

The purpose of this study was to identify the factor(s) responsible for the hypertrophic activity produced by cardiac fibroblasts in culture. Our strategy was to treat cardiac myocytes with conditioned medium from fibroblast cultures in the presence of antagonists to known hypertrophy factors. Several independent measures of hypertrophy were used to monitor the myocyte response. Our results show that cultured cardiac fibroblasts produce endothelin and LIF, which combine to generate the hypertrophic response.

Results

In order to enable rapid screening of a large number of samples, a visual rating sytstem was devised which indicates changes in the size of myocytes in serum-free culture in 96-well plates. Cell size is rated on an arbitrary scale of 1–7 by microscopic evaluation, referenced to the size increase induced by a maximal dose of phenylephrine, a well-characterized hypertrophic agent in the cultured rat myocyte system (Simpson et al., 1982). Untreated cells are assigned a rating of 3 relative activity units (RAU). Toxic effects are rated from 2 to 0 RAU. The positive control in each assay is cells treated with 100 μmol/L phenylephrine. These are assigned 7 RAU. Test samples are assigned code numbers and each assay is scored by two independent operators. Figure 1 illustrates the rating system and how it relates to myocyte surface area. In Fig. 1A crystal violet stained myocytes treated with increasing doses of phenyl-

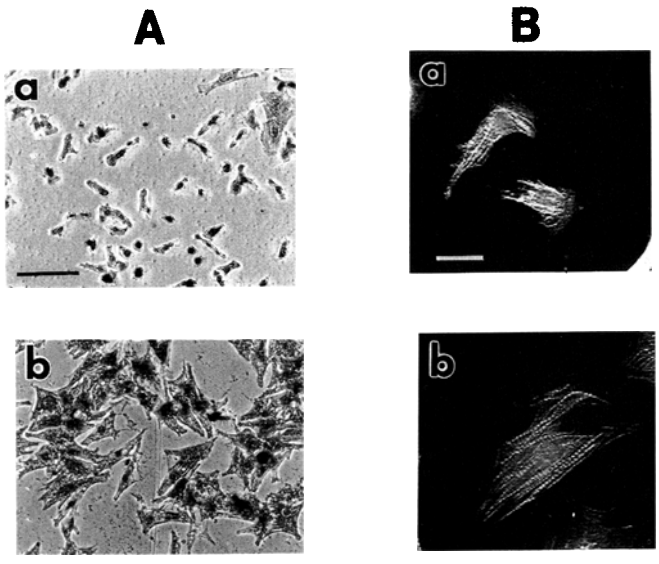

Fig. 2. Effect of rat neonatal cardiac fibroblast CM on the morphology and contractile fiber organization in cultured rat neonatal cardiac myocytes. Myocytes were treated with 50% (v/v) CM for 48 h. The nontreated control cells (a) and the treated cells (b) were stained with crystal violet to show gross morphology **(A)** or fluorescent phallacidin to show the f-actin in the contractile fibrils **(B)** (Panel A bar: 100 μm). (Panel B bar: 20 μm).

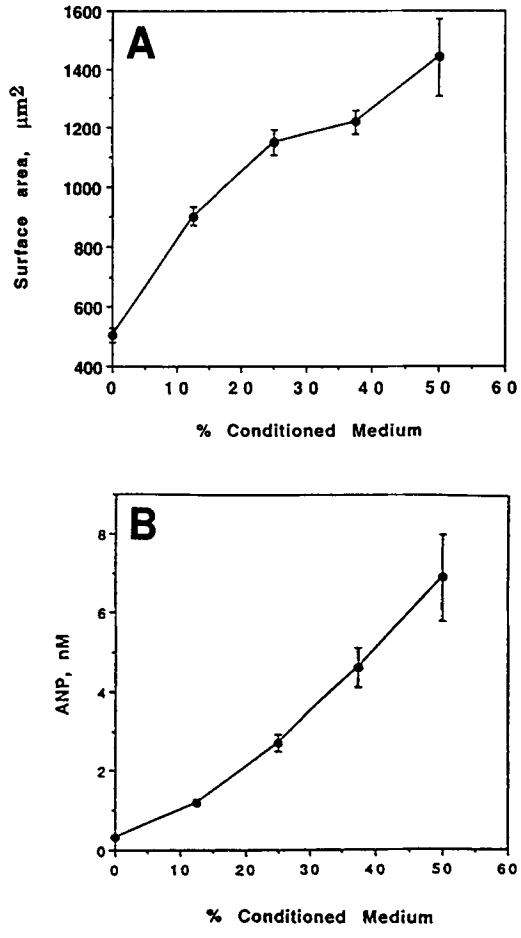

Fig. 3. Effect of rat neonatal fibroblast CM on cultured rat neonatal cardiac myocyte surface area and ANP production. Myocytes were treated with CM for 48 h. The culture medium was assayed for ANP **(B)**, and the cells were stained with crystal violet and measured for cell surface area **(A)**. The data represent the mean and standard error of two experiments done in duplicate and assayed in duplicate

ephrine and representative for each relative activity rating are shown, along with the mean surface area/cell for each photograph. Fig. 1B shows that the relative activity rating correlates with myocyte surface area (coefficient of correlation = 0.99). The increase in size that results from treatment of the myocytes with 100 μmol/L phenylephrine is not because of spreading only. The contractile fibrils, stained with a fluorescent probe for actin, have become elongated in parallel arrays (Fig. 1C). Additionally, after treatment of cultures with this dose of phenylephrine, myocyte protein/cell increases 40% over nontreated cells (data not shown). If test molecules are positive in this system, their effects on myocyte surface area, ANP production and protein/cell are then measured as quantitative indices of hypertrophy. Endothelin, serum, LIF, and cardiotrophin-1 (CT-1) have all been positive in this assay system.

Ventricular myocytes were exposed to fibroblast conditioned medium (CM), and hypertrophy was assessed by four independent assay endpoints: myocyte surface area, protein content/cell, organization of contractile fibrils, and production of ANP. Myocytes exposed to 50% (v/v) CM increased in size (Fig. 2A). Fluorescent staining of f-actin shows that there was a concomitant organization of the contractile units within the cells into parallel arrays (Fig. 2B). The increase in cell surface area was dose-dependent (Fig. 3A) and there was a 48% increase in protein content/cell (see Fig. 4D). ANP production by the ventricular myocytes also increased in a dose-dependent fashion with exposure to CM (Fig. 3B).

In order to determine whether any known hypertrophy agents might be present in CM, cultured myocytes were incubated with CM in the presence of antagonists and changes in myocyte size were determined by the rapid screening assay. This initial screen indicated that BQ-123, a specific antagonist to the endothelin-1 A receptor and a monoclonal antibody (mAb) to LIF, each partially reduced the increase in myocyte size (indicated in RAU) induced by 50% (v/v) CM. Both antagonists combined reduced myocyte size to near unstimulated levels (Fig. 4A). This observation was confirmed by surface area measurements (Fig. 4B). The increase in cellular protein content induced by CM was reduced in the presence of the combination of BQ123 and the LIF mAb to that of unstimulated myocytes (Fig. 4D). However, the reduction in protein/cell induced by the individual antagonists was not statistically significant given the variability of replicate measurements in this assay. The ANP production of the myocytes was also measured and the results in Fig. 4C show that the individual

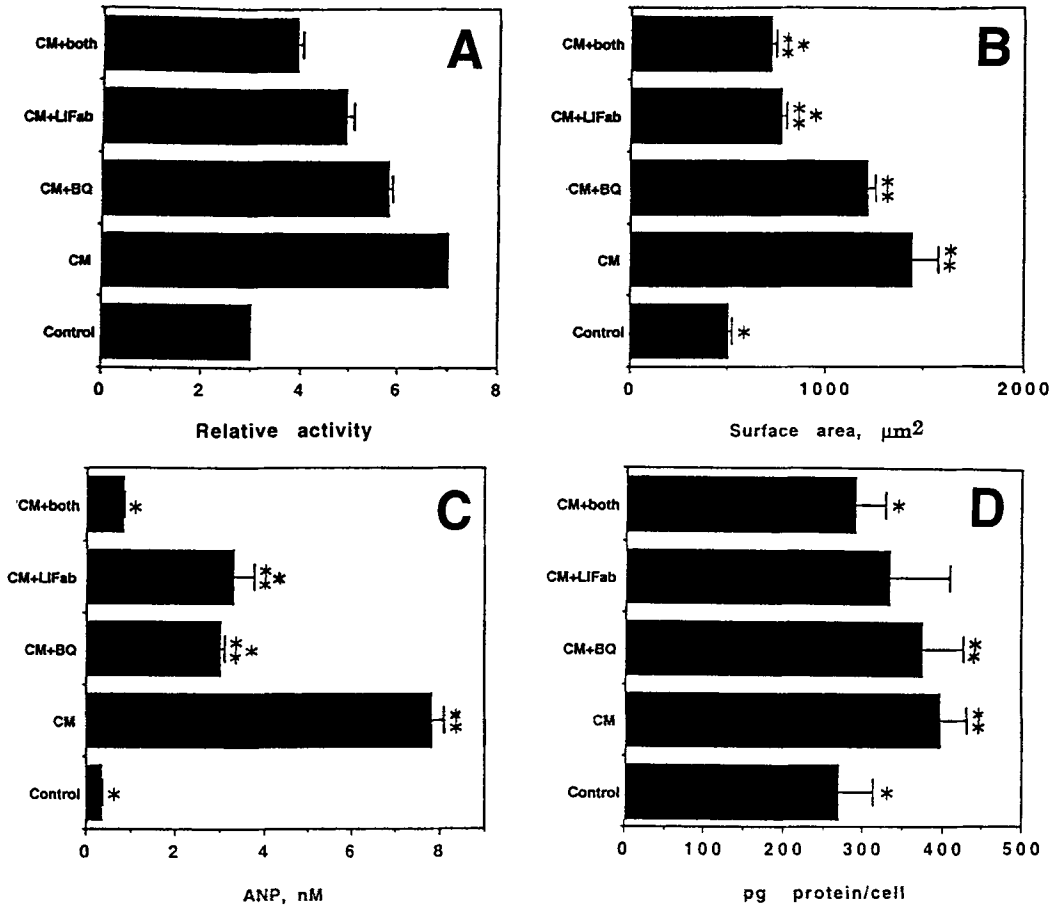

Fig. 4. Effect of ET and LIF inhibitors on fibroblast CM-treated myocytes. Myocytes (control) were treated with CM alone (50%, v/v) (CM), CM and 100 μmol/L BQ-123, CM and 50 μg/mL LIF mAb D62.3.2, CM and 100 μmol/L BQ-123 and 50 μg/mL LIF mAb D62.3.2 (CM+ both). The cells were stained, visually examined microscopically, and rated for relative activity, and measured for surface area **(A** and **B)** and the culture medium was assayed for ANP **(C)**. In separate cultures, the cells were removed from the culture surface, counted, and then assayed for protein content **(D)**. The data in all panels represent the mean and standard error. For the data in Panel A $n = 12$, for Panel B $n = 65$, for Panel C $n = 6$, for Panel D $n = 8$. Significance was determined by one way analysis of variance followed by the Bonferroni multiple comparisons test. $^*P < 0.01$ vs CM; $^{**}P < 0.01$ vs Control.

antagonists exert a partial inhibition of the response, whereas the mixture of antagonists results in complete inhibition. Figure 5 shows the morphology of the myocytes treated with CM alone and with BQ-123 and the LIF mAb. Treatment with CM in the presence of both antagonists (5f) resulted in cells which resemble the untreated control cells (5a).

BQ-123 and the LIF mAb were specific for their respective targets. Neither had an effect on unstimulated cells, nor did they inhibit myocyte hypertrophy induced by phenylephrine and CT-1 (data not shown). The results of these antagonist studies indicate that the hypertrophy activity in the CM is most likely derived from a combination of endothelin and LIF.

In order to confirm that LIF and endothelin were, in fact, present in CM, we looked for the mRNA for both molecules in the cells and for the proteins in the conditioned medium. Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were used to show that the transcripts for LIF and endothelin were present in a total RNA preparation obtained from the fibroblast culture. Oligonucleotide primers were designed to amplify 340- and 518-bp stretches of the coding sequences for LIF and endothelin, respectively. cDNA products were found at the predicted molecular weights (Fig. 6), and the identities of these transcripts were confirmed by sequence analysis.

Endothelin and LIF were detected in the CM by immunoassay. The concentration of endothelin (endothelin-1 and -2 and big endothelin) averaged 229 ± 10 pmol/L for three preparations. The concentration of rat LIF in the CM was estimated using a sandwich ELISA with two antihuman LIF monoclonal antibodies and mouse LIF as the standard. The value obtained for the concentration of LIF using this technique was 190 ± 50 pmol/L for three preparations.

Myocytes were treated with purified LIF and endothelin and the response was compared to that observed with the CM. LIF and endothelin produce distinctive morphological changes in the cells (Fig. 7A). Both ET-1 (Fig. 7) and big endothelin (data not shown) induced hypertrophy in myocyte cultures at concentrations as low as 10–25 p*M*. Myocytes exposed to LIF tend to be stretched with den-

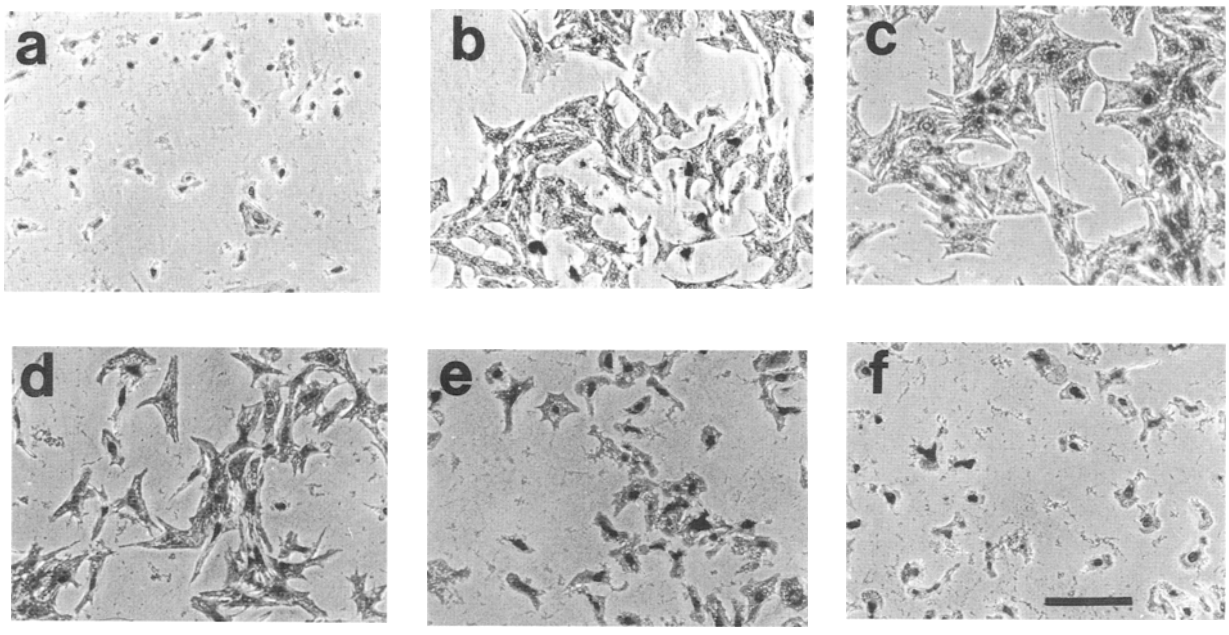

Fig. 5. Effect of ET and LIF inhibitors on the morphology of rat neonatal cardiac myocytes treated with CM. Myocytes were treated with assay medium alone **(a)**, 100 μmol/L phenylephrine **(b)**, 50% CM **(c)**, 50% CM and 100 μmol/L BQ-123 **(d)**, 50% CM and 50 μg/mL LIF mAb D62.3.2 **(e)**, and 50% CM and 100 μmol/L BQ-123, and 50 μg/mL LIF mAb D62.3.2 **(f)**. The myocytes were stained with crystal violet.

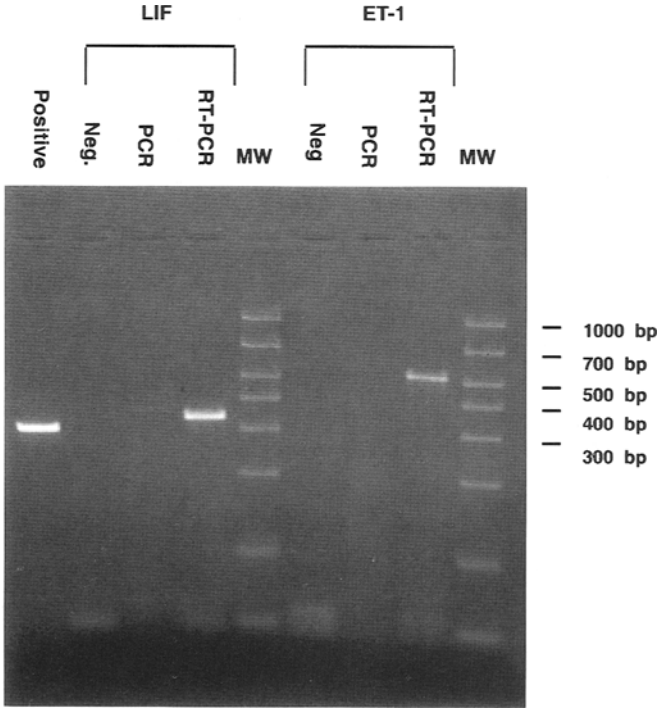

Fig. 6. RT-PCR of LIF and endothelin messages from RNA isolated from cardiac fibroblasts. RT-PCR reactions were analyzed by gel electrophoresis. The lane labeled "Positive" contains the kit RT-PCR positive control, expected size 308-bp. The lanes labeled LIF contain a negative control (no RNA added), PCR reaction only and RT-PCR using 150 ng total fibroblast RNA and the LIF primers. The expected product size for LIF is 340-bp. Similar reactions were done for endothelin (ET-1). The expected product size is 518-bp.

dritic processes. Those exposed to endothelin are more compact with no processes. Exposure to the combination results in a hybrid phenotype with a loss of processes and an increase in size that resembles the morphology resulting from treatment with phenylephrine or CM (*see* Fig. 5B,C).

The organization of the contractile fibrils is shown in Fig. 7B. The fibrils in the nontreated controls are disorganized and short compared to those in the myocytes that have hypertrophied. The fibrils in the endothelin-treated cells are not organized in parallel, have a tangled appearance, and do not show prominent banding. The fibrils in the myocytes treated with LIF, the combination of LIF and endothelin, and phenylephrine are organized in parallel arrays with prominent banding showing the sarcomeric units. In the LIF-treated cells the contractile fibrils can be seen to extend to the tips of the projections.

The dose-dependent effects of LIF, endothelin, and the combination of the two substances on ANP production are shown in Fig. 8. Individually, the two factors produce intermediate levels of ANP, whereas the combination of the two factors induce a high level of ANP production.

We performed immunocytochemistry studies to confirm that the nonmyocytes used in these studies were fibroblasts. The majority (99%) of the nonmyocytes stained with an antibody to smooth muscle actin in a stress fiber pattern (Fig. 9). There was less than 1% contamination with endothelial cells (as assessed with an antibody to Von Willebrand Factor), and myocytes (detected with an antibody to sarcomeric tropomyosin). There was no staining on control slides where the primary antibodies had been omitted.

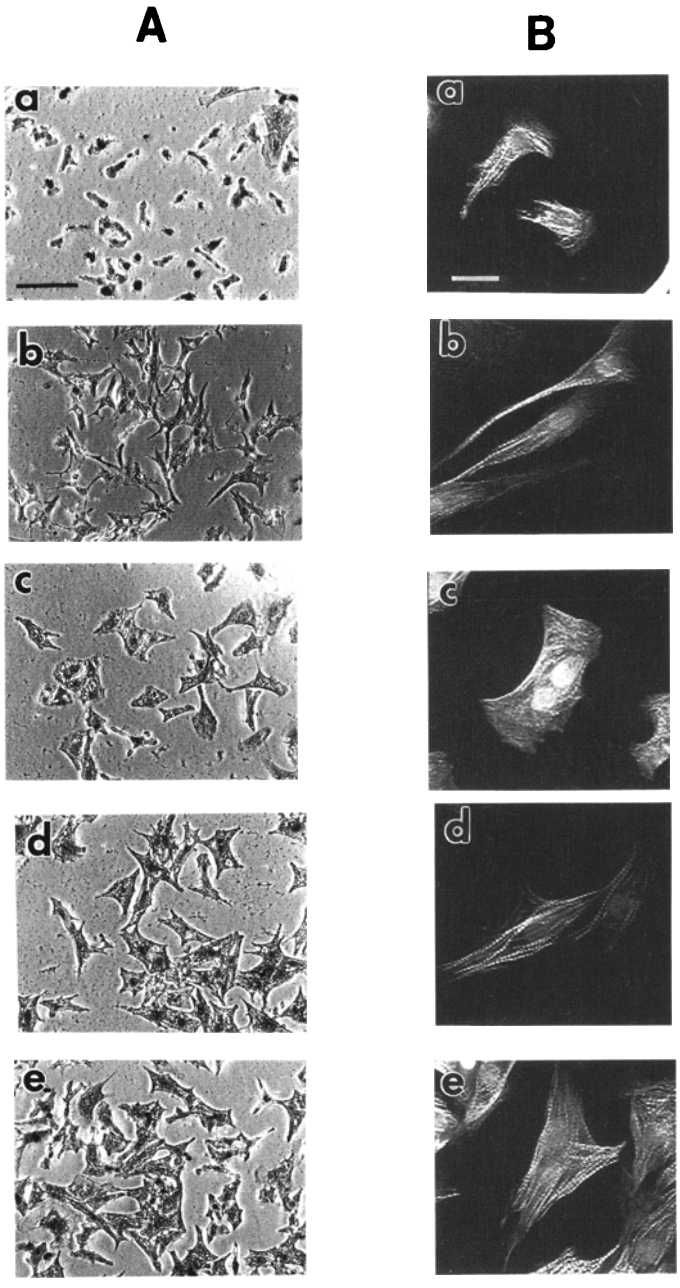

Fig. 7. Effect of LIF and endothelin on the morphology and contractile fiber organization of cultured neonatal rat cardiac myocytes. Myocytes were treated with assay medium alone **(a)**, 1 nmol/L mouse LIF **(b)**, 5 nmol/L endothelin-1 **(c)**, 1 nmol/L mouse LIF and 5 nmol/L endothelin-1 **(d)**, and 100 μmol/L phenylephrine **(e)**. The myocytes were stained with crystal violet to show gross morphology **(A)**, or fluorescent phallacidin to show f-actin in the contractile fibrils **(B)** (Panel A bar: 100 μm). (Panel B bar: 20 μm).

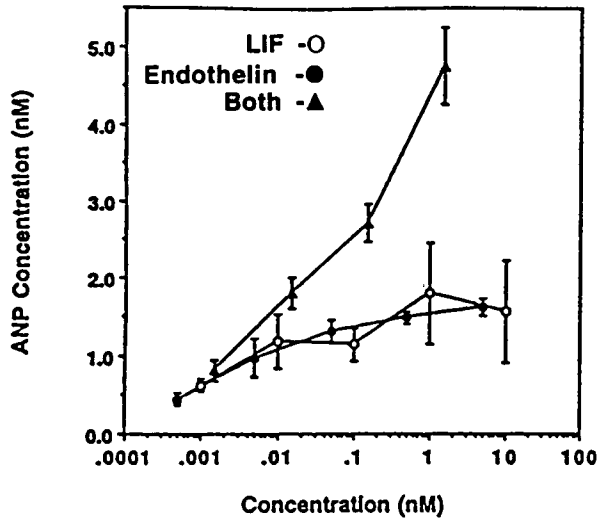

Fig. 8. Effect of LIF and endothelin on cultured neonatal rat cardiac myocyte ANP production. Myocytes were treated with varying concentrations of mouse LIF, endothelin-1, and a combination of mouse LIF and endothelin-1. The assay medium was assayed for ANP. The data represent the mean and standard error of three experiments done in duplicate and assayed in duplicate. The concentration of the combination of LIF and endothelin was determined by adding the concentrations of each agent.

Discussion

The results of this study confirm the observations made by Long et al. (1991), which show that conditioned medium from cultured cardiac fibroblasts contains factor(s) that induce cardiac myocyte hypertophy in vitro. We have identified those factors as LIF and endothelin. The transcripts for these substances were detected in RNA purified from the cells, and the proteins were detected in the CM. The hypertrophic activity in the CM was also inhibited by a combination of antagonists to these substances.

LIF is a polyfunctional cytokine that has been shown to suppress colony formation in a mouse myeloid leukemic cell line, prevent differentiation in normal embryonic stem cells, stimulate the production of acute phase proteins in hepatocytes, inhibit lipoprotein lipase, and affect bone formation (Metcalf, 1992). The first indication that LIF may directly affect the growth of cardiac myocytes came from studies that lead to the cloning of CT-1. CT-1 is a protein produced by differentiating mouse embryoid bodies and also induces cardiomyocyte hypertrophy in vitro (Pennica et al., 1995). CT-1 and LIF are members of a cytokine family that includes CNTF, oncostatin M, IL6, and IL11. Of these, LIF and CT-1 show the most potent hypertrophy activity on neonatal rat cardiac myocytes in culture. The role that LIF may play in cardiac physiology or disease is unknown.

Separate genes encode the three endothelin related peptides, endothelin-1, -2, and -3 (Inoue et al., 1989). Endothelin-1 is a 21 amino acid peptide that is a potent venous and arterial vasoconstrictor. The mature biologically active peptide is a proteolytic product of the 38–39 amino acid molecule "Big Endothelin" (Yanagisawa et al., 1989). Endothelin has been shown to affect the cells in the heart both in vivo and in vitro. In vivo endothelin is present in both atrial and ventricular myocardium in healthy and failing hearts and enhances myocardial inotropic activity, vascular smooth muscle proliferation, and coronary vasoconstriction (Wei et al., 1994). Studies in a rat model of left ventricular overload suggest that endothelin may act as an

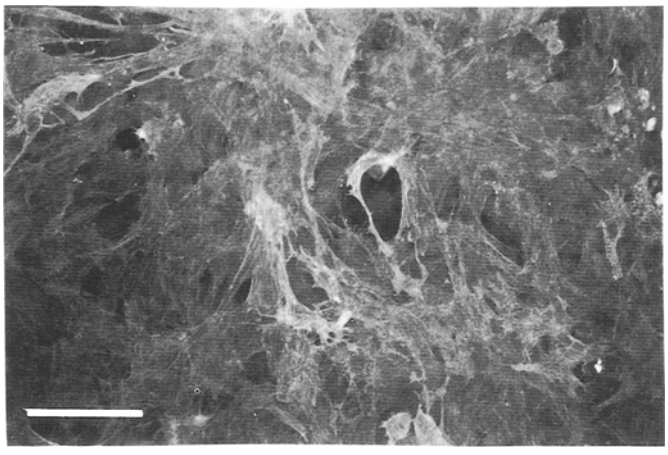

Fig. 9. Cultured neonatal rat cardiac fibroblasts stained with an antibody to alpha smooth muscle actin. Passage one fibroblasts were cultured on glass chamber slides for 5 d, then stained with an antibody to alpha smooth muscle actin followed by a fluorescein-conjugated secondary antibody (Bar: 100 μm).

initiating hypertrophy factor during the early phase of pressure overload (Itoh et al., 1991). In vitro endothelin stimulates multiple cell-signalling pathways in cultured adult cardiac myocytes (Jones et al., 1992; Hilal-Dandan et al., 1994) and induces hypertrophy in cultured neonatal myocytes (Shubieta et al., 1990; Ito et al., 1991; Suzuki et al., 1991).

Immunofluorescence studies indicate that the cultures used in this study contain primarily fibroblast-like cells (which stain with antibodies to smooth muscle actin) with less than 1% endothelial cells and myocytes. Brouty-Boye et al. reported alpha smooth muscle actin expression in cultured fibroblasts derived from newborn rat cardiac tissue (Brouty-Boye et al., 1992). The myocytes in their study also expressed this actin isoform and could be distinguished from the fibroblasts by the pattern of staining: stress fiber for fibroblasts vs a striated pattern for myocytes. A similar stress fiber-staining pattern was reported by Long et al. in their study of cardiac nonmyocytes (Long et al., 1991). It is likely that both the LIF and the endothelin in these cultures are being produced by the fibroblasts, since there are so few contaminating endothelial cells and myocytes. Several laboratories have shown that fibroblasts in culture can produce LIF (Hamilton et al., 1993; Elias et al., 1994; Lorenzo et al., 1994). Furthermore, endothelins are produced not only by vascular endothelial cells, but by epithelial cells, macrophages, fibroblasts, and many other types of cells (Battistini et al., 1993).

Myocytes exposed to LIF and endothelin develop morphologies distinct to each effector. The combination of these factors produces another distinct morphological phenotype. Neither LIF nor endothelin alone can generate the same level of ANP production as the combination of the two factors. Taken together these observations provide an experimental basis for the concept that net hypertrophy in vivo may be the result of stimulation by multiple factors.

We have identified LIF and endothelin as two important mediators of myocyte hypertrophy in CM, but cardiac fibroblasts may produce other factors as well. Labile substances may not be detectable using the protocols described here. It is also important to note that since only the conditioned medium from the fibroblasts was analyzed, this study does not address the possibility that these cells may secrete hypertrophy factors that remain bound to the cell surface or to the extracellular matrix.

Further evaluation is necessary to determine the relevance of this study to the mechanisms of physiologic and pathophysiologic cardiac hypertrophy in vivo. However, we have shown that cardiac fibroblasts have the potential to produce muliple hypertrophic factors, and their numbers and location in the heart make them ideally suited to modulate myocyte growth in a paracrine fashion.

Materials and Methods

Materials

The collagenase CLS 2 was from Worthington (Freehold, NJ) and the Percoll from Pharmacia Biotech AB (Uppsala, Sweden). The culture media and supplements were from Gibco-BRL (Grand Island, NY). The aprotinin and crystal violet were from Sigma. The crystallized BSA was from ICN Biomedicals (Aurora, IL). The Falcon 96 well plates were from Becton Dickenson (Oxnard, CA) and the Lab Tek chamber slides from Nunc (Naperville, IL). The Micro BCA Protein Assay Reagent was from Pierce (Rockford, IL). Human/porcine endothelin-1 was from American Peptides (Sunnyvale, CA). The ^{125}I-ANP was from Amersham (Arlington Heights, IL). The recombinant murine LIF, the antihuman LIF monoclonal antibodies (Kim et al., 1992), the BQ-123 (*see* Webb et al., 1992), the rat ANP and the rat ANP-receptor IgG fusion protein were made at Genentech.

Myocyte Culture

Neonatal rat cardiac ventricular myocytes were cultured in 96-well plates as previously described in Pennica et al. (1995). This culture system is a modification of a well-characterized system (Iwaki et al., 1990) in order to accomodate a larger test sample number and a small test sample volume. The exposure to serum was reduced from 24 h to less than 30 min to increase the sensitivity of the myocytes to test substances since serum induces hypertrophy. Myocytes were isolated from 1-d-old Sprague-Dawley rats by a series of collagenase digestions followed by Percoll gradient purification (Iwaki et al., 1990). The myocytes, which band at the lower gradient interface, were collected, washed twice and resuspended in F12/DME medium with 15% (v/v) fetal calf serum to inactivate any toxic residuals from the gradient purification. After 10–30 min the cells were diluted into serum-free F12/DME medium supplemented with 10 μg/mL transferrin, 1 μg/mL insulin, 1 μg/mL aprotinin, 2 mmol/L glutamine, 100 U/mL penicillin G, and

100 μg/mL streptomycin (assay medium). The final serum concentration of this assay medium with cells was <0.1%. Myocytes were plated 200 μL/well at a density of 7.5×10^4 cells/mL in 96-well flat-bottomed plates that had previously been coated with F12/DME medium with 4% (v/v) fetal calf serum for 8 h at 37°C. Random field counts of six different preparations of plated cells indicated that 85–90% of these cells were myocytes. After 24 h at 37°C in 5% CO_2, test substances were added. Forty-eight hours after the test substances were added, the culture medium was removed for ANP measurement, and the cells were fixed and stained with 0.5% (w/v) crystal violet in methanol and formaldehyde. This procedure facilitates the microscopic examination of the cells and provides a permanent record of the results.

Fibroblast Culture

The band at the upper gradient interface is enriched for fibroblasts in the procedure described in the section "Myocyte Culture." They were collected, washed twice and resuspended in F12/DME medium with 10% fetal calf serum (30 mL/50 hearts) and plated in T75 flasks (2/50 hearts). After 1 h at 37°C in 5% CO_2, the flasks were gently swirled and the medium replaced. After 4 d in culture the cells were trypsinized and replated at 4×10^5 cells/mL in T25 flasks (5 mL/flask). Almost all of the contaminating myocytes are destroyed by this procedure. After 5 d in culture, the cells were washed twice in serum-free F12/DME medium and conditioning medium (myocyte hypertrophy assay medium with 1 mg/mL BSA) was added. The conditioned medium was removed after 24 h, centrifuged to remove cells and debris and stored at 4°C.

Myocyte Surface Area Measurment

Myocyte surface areas were measured from digitized images of crystal violet-stained cells using the computer program NIH Image 1.57. The number of myocytes measured for each data point was 50 or greater.

ANP Measurements

Rat ANP concentrations were determined by competition for the binding of rat 125I-ANP for a rat ANP receptor A-IgG fusion protein (Bennett et al., 1991). Rat ANP receptor A-IgG fusion protein was added to the wells of a 96-well microtiter plate that had been precoated with an affinity purified F(ab')2 fragment specific for the Fc region of human IgG (Jackson Immuno Research, West Grove, PA). The plates were incubated for 1 h at room temperature with gentle agitation, washed and test samples were added. The plates were incubated at room temperature for 1 h with gentle shaking, then rat ^{125}I-ANP was added. After incubation for 1 h at room temperature with gentle shaking, the wells were aspirated and washed. Microscint-20 (Packard, Meridien, CT) was added to each well for 15 min with shaking. Radioactivity was measured using a Packard Topcount. The amount of ANP in each sample was calculated from a standard curve of rat ANP.

Protein/Cell Measurements

Myocytes were plated onto 12-well plates (Costar/Corning, Corning, NY) precoated with 4% fetal calf serum in F12/DME medium for 8 h at 37°C at a density of 1.5×10^5 cells/mL, 2 mL/well. After 24 h at 37°C in 5% CO_2, test substances were added. Forty eight h later the medium was removed, the cells washed three times with phosphate-buffered saline (PBS), trypsinized, resuspended in 5% fetal calf serum in PBS, centrifuged and washed once with PBS. After resuspension in PBS an aliquot was removed for counting. Sodium dodecyl sulfate was added to the remaining suspension to a final v/v of 1%. Protein was assayed according to manufacturer's directions with a Pierce Micro BCA Protein Assay Reagent.

Endothelin Measurements

Endothelin concentrations were determined with the Amersham Endothelin 1,2 (high sensitivity) assay system.

LIF Measurements

The LIF sandwich ELISA was performed as previously described in Kim et al. (1992) with the following modifications. After the microtiter plates were coated overnight with monoclonal antibody D4.16.9 (to human LIF), blocked with 0.5% (w/v) BSA and washed, the murine LIF standards and samples were added and the plates incubated for 2 h at room temperature. Then biotinylated (with Pierce ImmunoPure Sulfo-NHS-Biotin) monoclonal antibody D62.3.2 was added and the plates incubated at room temperature for 1 h. The plates were washed and strepavidin-peroxidase conjugate (Boehinger Mannheim Biochemicals, Indianapolis, IN) was added and the plates incubated for 30 min at room temperature. The plates were washed and the peroxidase substrate TMB (tetramethylbenzidine) (Kirkegaard and Perry, Gaithersburg, MD) was added. Color development was stopped after 10 min by the addition of H_3PO_4. The absorbance at 450 nm was determined using a microtiter plate reader. An estimate of the concentration of the rat LIF in the CM was determined by comparison to a murine LIF standard curve.

RNA Isolation and RT-PCR for LIF and Endothelin Message

RT-PCR was performed for detection of rat endothelin and LIF mRNA (Perkin Elmer kit #N808-0069). Reactions contained 150 ng of total RNA prepared from rat neonatal fibroblasts using the RNA STAT-60 kit (Tel-Test "B," Inc.). Primers were designed to rat endothelin (et-1 forward 5' TCTCTGCTGTTTGCTTTCC 3'; et-1 reverse 5' AAGGAGGTCTTGCTGTTGC 3') and leukemia inhibitory factor; LIF (lif forward 5' CCTTCCCATCACCCCTGTAAAT 3'; lif reverse 5' GAGTTTGATCTGGAGGCTCACG 3'). Reverse transcription was performed as/the protocol holding at 50°C for 5 min, 55°C for 5 min, and 60°C for 15 min. Next PCR was carried out for 35 cycles of 94°C/55°C/72°C. One control reaction for each primer set was per-

formed with the PCR step only (this was to confirm the products observed resulted from mRNA targets). The negative control RT-PCR reactions did not contain the total RNA. After RT-PCR the reactions were analyzed on a 3% 3:1 NuSievE agarose gel (FMC BioProducts) and visualized with ethidium bromide UV-fluorescence. The authenticity of the endothelin and LIF RT-PCR products were confirmed by sequencing.

Fluorescent Staining

For actin staining the myocytes were plated in 4-chamber Lab Tek glass chamber slides precoated for 8 h with 4% (v/v) fetal calf serum in F12/DME medium and cultured for 24 h. They were exposed to test substances for 48 h and then washed three times in PBS and fixed in 95% (v/v) ethanol for 15 min. The slides were then washed three times in PBS with 0.1% (v/v) Tween-20, permeabilized using PBS with 0.3% Triton X-100 for 2 min, and washed three times with 0.1% Tween-20. The slides were incubated with phallacidin conjugated to BODIPY FL (10 μg/mL in 1% BSA in PBS) (Molecular Probes, Eugene, OR) for 40 min to stain the f-actin present in the contractile fibrils. The slides were then washed 3X with PBS with 0.1% Tween-20 and once with water. Images of the phallacidin-stained cells were acquired on a Ultima laser scanning confocal microscope (Meridian Instruments, Okemos, MI). A 1.4 NA 60X oil immersion objective was utilized coupled with 488-nm excitation, with the resulting fluorescence measured following a 525-nm long-pass filter. Data were collected at a horizontal and vertical resolution of 0.2 μm and a z-resolution of 0.5 μm. Each data point was collected as an average of 200 measurements. The final images were constructed with a maximum fluorescence projection algorithm by compressing the multiple z or depth images into a single two-dimensional representation.

For cell-specific marker staining, the fibroblasts were plated in 4-chamber Lab Tek glass chamber slides, cultured for 5 d, washed three times with PBS, and fixed in 95% ethanol for 15 min. The slides were washed three times in PBS, blocked for 30 min with 1% BSA in PBS with 0.1% (v/v) Triton X-100, and incubated with the following primary antibodies for 2 h: monoclonal antitropomyosin (sarcomeric) (Sigma, St. Louis, MO) 1:50 to stain for myocytes, monoclonal antialpha smooth muscle actin (Sigma) 1:2500 to stain for fibroblast-like cells, and rabbit antihuman Von Willebrand Factor (DAKO, Carpenteria, CA) 1:500 to stain for endothelial cells. After three washes with PBS, the slides were incubated with the appropriate fluorescein-conjugated secondary antibodies (Sigma) 1:200 for 45 min and washed three times with PBS.

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